# DEVICE AND METHOD FOR HIGH-THROUGHPUT QUANTIFICATION OF MRNA FROM WHOLE BLOOD

## Background of the Invention

### Related Applications

**[0001]** This application is a continuation-in-part of international application No. PCTUS03/12895, which was filed in English and expected to be published in English, and claims the benefit of provisional Application No. 60/375472.

### Field of the Invention

**[0002]** The present invention relates to high-throughput isolation and quantification of mRNA from whole blood. More particularly, this invention relates to a method and device for isolating and amplifying mRNA using combinations of leukocyte filters attached to oligo(dT)-immobilized multi-well plates.

### Description of the Related Art

**[0003]** Research in the field of molecular biology has revealed that the genetic origin and functional activity of a cell can be deduced from the study of its ribonucleic acid (RNA). This information may be of use in clinical practice, to diagnose infections, to detect the presence of cells expressing oncogenes, to detect familial disorders, to monitor the state of host defense mechanisms and to determine the HLA type or other marker of identity. RNA exists in three functionally different forms: ribosomal RNA (rRNA), transfer RNA (tRNA) and messenger RNA (mRNA). Whereas stable rRNA and tRNA are involved in catalytic processes in translation, mRNA molecules carry genetic information. Only about 1-5% of the total RNA consists of mRNA, about 15% of tRNA and about 80% of rRNA.

**[0004]** mRNA is an important diagnostic tool, particularly when it is used to quantitatively observe up- or down-regulation of genes. Human peripheral blood is an excellent clinical resource for mRNA analysis. The detection of specific chimeric mRNA in blood, for example, indicates the presence of abnormal cells and is used in molecular diagnostics for chronic myelogenous leukemia (CML) (Kawasaki E.S., Clark S.S., Coyne M.Y., Smith S.D., Champlin R., Witte O.N., and McCormick F.P. 1988. Diagnosis of chronic myeloid and acute lymphocytic leukemias by detection of leukemia-specific mRNA

sequences amplified *in vitro*. Proc. Natl. Acad. Sci. USA 85:5698-5702, Pachmann K., Zhao S., Schenk T., Kantarjian H., El-Naggar A.K., Siciliano M.J., Guo J.Q., Arlinghaus R.B., and Andreeff M. 2001. Expression of bcr-able mRNA individual chronic myelogenous leukaemia cells as determined by in situ amplification. Br. J. Haematol. 112:749-59). Micrometastatic cancer cells can also be detected in blood by measuring cancer-specific mRNA, such as carcinoembryonic antigen (CEA) for colon cancer, prostate specific antigen (PSA) for prostate cancer, thyroglobulin for thyroid cancer (Wingo S.T., Ringel M.D., Anderson J.S., Patel A.D., Lukes Y.D., Djuh Y.Y., Solomon B., Nicholson D., Balducci-Silano P.L., Levine M.A., Francis G.L., and Tuttle R.M. 1999. Quantitative reverse transcription-PCR measurement of thyroglobulin mRNA in peripheral blood of healthy subjects. Clin. Chem. 45:785-89), and tyrosinase for melanoma (Pelkey T.J., Frierson H.F. Jr., and Bruns D.E. 1996. Molecular and immunological detection of circulating tumor cells and micrometastasis from solid tumors. Clin. Chem. 42:1369-81). Moreover, as the levels of these cancer-specific mRNA can change following treatment, quantification of specific mRNA provides for a useful indicator during treatment follow-up.

**[0005]** As blood contains large quantities of non-nucleated erythrocytes (approximately 5 million cells/μL) compared to leukocytes (approximately 5000 leukocytes/μL), the isolation of granulocytes or lymphocytes from whole blood is commonly performed as the first step in mRNA analysis. However, due to inconsistencies in the recovery of specific subsets of leukocytes among different samples, the number of isolated leukocytes is determined for each sample and results are expressed as the quantity of mRNA per leukocytes, not mRNA/μL blood. Moreover, mRNA quantities may change during lengthy isolation processes. While no method exists for the isolation of cancer cells from blood, gene amplification technologies enable the identification and quantification of specific mRNA levels even from a pool of different genes, making whole blood an ideal material for mRNA analysis when gene-specific primers and probes are available.

**[0006]** It is very difficult to isolate pure mRNA from whole blood because whole blood contains large amounts of RNAases (from granulocytes) and non-nucleated erythrocytes. Although various RNA extraction methods are available for whole blood applications (de Vries T.J., Fourkour A., Punt C.J., Ruiter D.J., and van Muijen G.N. 2000.

Analysis of melanoma cells in peripheral blood by reverse transcription-polymerase chain reaction for tyrosinase and MART-1 after mononuclear cell collection with cell preparation tubes: a comparison with the whole blood guanidinium isothiocyanate RNA isolation method. Melanoma Research 10:119-26, Johansson M., Pisa E.K., Tormanen V., Arstrand K., and Kagedal Bl. 2000. Quantitative analysis of tyrosinase transcripts in blood. Clin. Chem. 46:921-27, Wingo S.T., Ringel M.D., Anderson J.S., Patel A.D., Lukes Y.D., Djuh Y.Y., Solomon B., Nicholson D., Balducci-Silano P.L., Levine M.A., Francis G.L., and Tuttle R.M. 1999. Quantitative reverse transcription-PCR measurement of thyroglobulin mRNA in peripheral blood of healthy subjects. Clin. Chem. 45:785-89), the assay procedures are labor-intensive, require several rounds of centrifugation, and involve careful handling that is essential in eliminating ribonuclease activities.

**[0007]** Consequently, there exists a need for a quick and easy method and device for isolating and quantifying large quantities of mRNA from whole blood. Specifically, there exists a need for a high throughput, whole blood-derived mRNA-processing technology with reproducible recovery and a seamless process to gene amplification.

## Summary of the Invention

**[0008]** The present invention discloses an efficient high throughput method and device for isolating and quantifying mRNA directly from whole blood, with reproducible recovery, using combinations of leukocyte filters attached to oligo(dT)-immobilized multi-well plates.

**[0009]** One aspect of the invention includes a method of high throughput quantification of mRNA in whole blood, including the steps of: (a) collecting whole blood; (b) removing erythrocytes and blood components from the whole blood by filtration to yield leukocytes on a filter membrane; (c) subjecting the leukocytes to cell lysis to produce a lysate containing mRNA; (d) transferring the lysate to an oligo(dT)-immobilized plate to capture the mRNA; and (e) quantifying the mRNA.

**[0010]** In one preferred embodiment of the method, an anticoagulant is administered to the whole blood prior to collection of leukocytes. Several filter membranes can be layered together to increase the yield of captured leukocytes. The leukocytes that are trapped on the filter membrane are lysed using a lysis buffer to release mRNA from the

leukocytes. The transfer of lysate to the oligo(dT)-immobilized plate can be accomplished using centrifugation, vacuum aspiration, positive pressure, or washing with ethanol followed by vacuum aspiration. The mRNA is quantified by producing cDNA and amplifying the cDNA by PCR.

**[0011]** Another aspect of the invention includes a device for performing high throughput quantification of mRNA in whole blood, wherein the device includes: (a) a multi-well plate containing: a plurality of sample-delivery wells; a leukocyte-capturing filter underneath the wells; and an mRNA capture zone underneath the filter which contains immobilized oligo(dT); and (b) a vacuum box adapted to receive the filter plate to create a seal between the plate and the box. In one preferred embodiment of the device, the leukocytes are captured on a plurality of filter membranes that are layered together. In another preferred embodiment of the device, the vacuum box is adapted to receive a source of vacuum. In another preferred embodiment of the device, a multi-well supporter is inserted between the vacuum box and the multi-well plates.

**[0012]** Another aspect of the invention includes a kit, which contains: the device for performing high throughput quantification of mRNA in whole blood, heparin, a hypotonic buffer, and a lysis buffer.

**[0013]** Another aspect of the invention includes a fully automated system for performing high throughput quantification of mRNA in whole blood, including: a robot to apply blood samples, hypotonic buffer, and lysis buffer to the device; an automated vacuum aspirator and centrifuge, and automated PCR machinery.

## Brief Description of the Drawings

**[0014]** FIG. 1 is an exploded drawing of the high throughput mRNA device.

**[0015]** FIG. 2 depicts the multi-well plate, including the leukocyte filter and oligo-(dT)-immobilized filter wells, of the high throughput mRNA device.

**[0016]** FIG. 3 is a graph showing the efficiency of leukocyte trapping of fresh and frozen blood samples on filter plates.

**[0017]** FIG. 4 is a graph showing the effect of number of washes of blood on mRNA quantification.

[0018] FIG. 5 is a graph showing the effect of final treatments of filter plates before cell lysis on mRNA quantification.

[0019] FIG. 6 is a graph showing how lysis buffer inhibits RNase.

[0020] FIG. 7 is a graph showing optimal concentrations of reverse transcriptase for mRNA quantification.

[0021] FIG. 8 is a graph showing optimal values of cDNA for PCR to capture mRNA.

[0022] FIG. 9 is a graph showing the hybridization kinetics of the invention.

[0023] FIG. 10 is a graph showing the linear relationship between whole blood volume used per well and mRNA quantification.

[0024] FIG. 11 is a graph showing optimal guanidine thiocyanate concentration.

[0025] FIG. 12 is a graph showing optimal proteinase K concentration.

Detailed Description of the Preferred Embodiment

[0026] The present invention allows analysis of larger volumes of unprepared whole blood, provides an efficient means of analyzing mRNA that is derived exclusively from white blood cells; removes rRNA and tRNA, provides consistent mRNA recovery, and is easily adaptable to automation. The present invention provides a sensitive quantification system, including: absolute quantification using real time PCR, and excellent reproducibility with coefficients of variation ranging from 20-25%. Moreover, the present invention is applicable to various disease targets (Table I).

[0027] **Table I. Clinical targets**

| Stimulation | Diseases | Candidate Genes |
|---|---|---|
| (-) | Leukemia | Translocation gene |
| | Cancer (diagnostics, monitoring, screening) | Cancer-specific gene from micrometastatic cancer cells |
| | HIV/CMV (diagnostics, monitoring, blood bank) | Virus-derived mRNA from infected WBCs |
| In vivo sensitivity | Anti-leukemia drugs | Apoptosis |
| | Immuno-suppressant | Cytokines |
| | Side effect of anti-cancer drugs on WBCs | Housekeeping genes |
| In vitro | Anti-viral drug sensitivity | Virus-derived mRNA from infected WBCs |

**[0028]** The invention is not limited to any particular mechanical structure. However, FIGs 1 and 2 show a preferred structure for implementing the high throughput mRNA quantification of the present invention. A vacuum box **10** forms the base of the structure. The vacuum box can be made of any material sufficiently strong to withstand vacuum aspiration; however, disposable plastic material is preferred. The vacuum box is adapted to receive a source of vacuum in order to perform vacuum aspiration **12**. A filter plug **14** is located within the vacuum aspirator adapter of the vacuum box. The vacuum box **10** preferably has a ledge **16** to mate with a multi-well filter plate **40**, or optionally, a multi-well supporter **20**. The multi-well supporter **20** is optionally provided inside the upper part of the vacuum box so as to support the multi-well filterplate **40**. A sealing gasket **30**, preferably comprised of silicon-based rubber or other soft plastic, is located on top of the multi-well supporter. Above the sealing gasket lies the multi-well filter plate **40**, which contains multiple sample wells **46**, multiple leukocyte-capturing filters **42** underneath the sample-delivery wells, and an mRNA capture zone **44** under the filter. Oligo(dT)-immobilized is contained in the wells of the mRNA capture zone.

**[0029]** One preferred embodiment involves a simple, reproducible, and high throughput method of mRNA quantification from whole blood. The rapid protocol minimizes the secondary induction or degradation of mRNA after blood draw, and the use of 96-well filterplates and microplates allows the simultaneous manipulation of 96 samples. Minimal manipulation during the procedure provides for very small sample-to-sample variation, with coefficient of variation (CV) values of less than 30%, even when PCR is used as a means of quantification.

**[0030]** In one embodiment, the method involves preparation of the vacuum box. In one preferred embodiment, a blood encapsulator such as polyacrylate polymer matrix (Red Z, Safetec) is added to the vacuum box to solidify the blood. A multi-well supporter is then placed in the vacuum box. A sealing gasket made of silicon-based rubber or other soft plastics is then placed on top of the multi-well plate supporter. A filter plug (X-6953, 60 μ Filter Plug HDPE, Porex Products Groups) is placed in the vacuum aspirator adapter of the vacuum box.

**[0031]** In this embodiment, the method involves the preparation of the filter plate. Either glassfiber membranes or leukocyte filter membranes can be used to capture leukocytes. In order to simplify the assay, multiple-well filterplates are constructed using glassfiber membranes or leukocyte filter membranes to enable the simultaneous processing of multiple blood specimens. Examples of filters for capturing leukocytes are disclosed in U.S. patent numbers 4,925,572 and 4,880,548, the disclosures of which are hereby incorporated by reference. Adsorption of leukocytes on fiber surfaces is generally accepted as the mechanism of leukocyte removal. Since the surface area of a given weight of fibers is inversely proportional to the diameter of the fibers, it is to be expected that finer fibers will have higher capacity and that the quantity as measured by weight of fibers necessary to achieve a desired efficiently will be less if the fibers used are smaller in diameter. A number of commonly used fibers, including polyesters, polyamides, and acrylics, lend themselves to radiation grafting, as they have adequate resistance to degradation by γ-radiation at the levels required for grafting and are of a structure with which available monomers can react. PBT has been the principal resin used for the development of the products of this invention and is the resin used in the examples. It should be noted, however, that other resins may be found which can be fiberized and collected as mats or webs with fibers as small as 1.5 micrometers or less, and that such products, with their critical wetting surface tensions adjusted as necessary to the optimum range, may be well suited to the fabrication of equally efficient but still smaller leukocyte depletion devices. Similarly, glass fibers, appropriately treated, may be usable to make effective devices. Absorption of CD4 mRNA is up to four times as effective when using PBT-based filters as opposed to glass fiber-based filters. The filter plate is placed in the vacuum box. In another preferred embodiment, multiple filter membranes are layered together to increase the amount of leukocytes captured from whole blood. In one preferred embodiment, the filter plate is placed upon the plate supporter and the sealing gasket. In another preferred embodiment, the filter plate is sealed with a plastic adhesive tape (Bio-Rad 223-9444), and the tape is cut to allow access to a desired number of wells. In another preferred embodiment, each well to which a sample will be added is washed with a hypotonic buffer (200 μL 5 mM Tris, pH 7.4).

**[0032]** The method preferably involves collecting blood, adding the blood to the multi-well filter plate, and removal of erythrocytes and other non-leukocyte components. In one preferred embodiment, whole blood can be drawn into blood collection tubes containing anticoagulants, which increase the efficiency of the leukocyte filtering. The anticoagulant, heparin, is particularly effective in increasing the efficiency of leukocyte filtering. In one preferred embodiment, the blood sample can be frozen, which removes some of the RNAases that destroy mRNA. The wells can be washed with a hypotonic buffer. Once blood has been added to the desired number of wells on the filterplate, the blood is filtered through the filter membrane. Filtration can be affected through any technique known to those of skill in the art, such as centrifugation, vacuum aspiration, or positive pressure.

**[0033]** In one especially preferred embodiment, vacuum aspiration is commenced (with 6 cm Hg) after the blood samples have been added to the filterplate wells. Each well is washed several times with a hypotonic buffer (12x with 200 μL 5 mM Tris, pH 7.4). In another preferred embodiment, each well containing a sample is washed with ethanol (1x with 200 μL 100% ethanol), which dries the filter membrane and significantly increases the efficiency of leukocyte trapping during vacuum aspiration. In another preferred embodiment, the vacuum is then applied (20 cm Hg for >2 min).

**[0034]** The method involves cell lysis and hybridization of mRNA to the oligo(dT)-immobilized within the mRNA capture zone. Lysis buffer is applied to the filterplate wells (40μL/well), and incubation is allowed to occur (room temperature for 20 min) to release mRNA from the trapped leukocytes. In one preferred embodiment, the multi-well filterplate is sealed in a plastic bag and centrifuged (IEC MultiRF, 2000 rpm, at 4 C, for 1 min). Lysis buffer is then added again (20 μL/well), followed by centrifugation (IEC MultiRF, 3000 rpm, at 4 C, for 5 min). The multi-well filterplate is then removed from the centrifuge and incubated (room temperature for 2 hrs).

**[0035]** In accordance with a preferred embodiment, the lysis buffer comprises a detergent, a salt, a pH buffer, guanidine thiocyanate, and proteinase K.

**[0036]** Preferred embodiments of the lysis buffer contain at least one detergent, but may contain more than one detergent. Those skilled in the art may utilize different combinations of concentrations of detergents with different strengths in order to achieve

varying levels of lysis of different membranes for various types of cells. For example, IGEPAL CA-630 is a weaker detergent than N-laurosarcosine, and in one embodiment IGEPAL CA-630 alone may be sufficient to lyse a cytoplasmic membrane. In other embodiments, a strong detergent, such as N-laurosarcosine can be used in combination with one or more weak detergents to optimize lysis of nuclear membranes. The detergents are preferably sufficient to lyse at least the cytoplasmic membrane of cells. Another preferred embodiment comprises a detergent sufficient to lyse the nuclear membrane of cells, as significant amounts of mRNA reside in the nuclei of cells. In some circumstances it is desirable to measure only cytoplasmic mRNA, while in other circumstances, it may be desirable to measure mRNA in the cytoplasm and nucleus.

**[0037]** Strong detergents of the lysis buffer preferably include, but are not limited to: N-lauroylsarcosine, S.D.S., Sodium deoxycholate, and Hexadecyltrimethylammonium bromide.

**[0038]** Weak detergents include IGEPAL CA-630, N-Decanoyl-N-methylglucamine, Octyl-β-D-glucopyranoside, or other detergents known to those skilled in the art. 0.05-2% detergent can be used in the lysis buffer. One particularly preferred embodiment of the lysis buffer includes 0.5% N-lauroylsarcosine. Another preferred embodiment of the lysis buffer contains 0.1-2% IGEPAL CA-630. A particularly preferred embodiment contains 0.1% IGEPAL CA-630.

**[0039]** The combination of salt and chelating agents also serve as a lysing agent. For example, 75 μM NaCl and 24 μM Na-EDTA can serve as a lysing agent. Embodiments of lysing agents may include other lysing agents known to those skilled in the art.

**[0040]** The salt of the lysis buffer acts as an mRNA-oligo(dT) hybridizing agent. The salt should preferably have a stringency (the rigor with which complementary DNA sequences hybridize together) that does not exceed that of 4X SSC, as determinable by those skilled in the art. Other embodiments of the lysis buffer include NaCl or other salts known to those skilled in the art.

**[0041]** The pH buffer of the lysis buffer stock preferably maintains a pH of 7.0-8.0. One embodiment comprises 1 mM-100 mM Tris HCl, pH 7.4. In a particularly preferred embodiment, the pH buffer comprises 10 mM Tris HCl, pH 7.4. Other preferred

embodiments of the lysis buffer include pH buffers known to those skilled in the art, including 0.1 M Citrate-Phosphate, pH 5.0, with 0.03% $H_2O_2$.

**[0042]** In accordance with a particularly preferred embodiment of the lysis buffer, guanidine thiocyanate serves as an RNAase deactivating agent. We have discovered that guanidine thiocyanate has typically been used in the prior art at insufficient concentrations to be effective. Therefore, preferably, the concentration of guanidine thiocyanate is greater than 1.4 M. Guanidine thiocyanate concentration as high as 10 M, more preferably no higher than 2 M can be used. However, as seen in **FIG 11**, at concentrations above 1.7 M, the efficiency of the lysis buffer is decreased. Accordingly, the preferred embodiment uses about 1.4 to about 1.75 M guanidine thiocyanate. One preferred embodiment comprises 1.7-1.8 M guanidine thiocyanate. A working lysis buffer can be prepared from the stock, as demonstrated in Example 4 below, with the particular concentration of 1.791 M guanidine thiocyanate. As other reagents are added to the lysis buffer, the concentration of guanidine thiocyanate becomes diluted. Where 55 ml of other reagents are added to 1 ml of the buffer as in Example 4, the preferred lysis buffer comprises guanidine thiocyanate in concentrations of about 1.61 to about 1.71 M. Thus, a preferred embodiment comprises guanidine thiocyanate in concentrations of about 1.6 to about 1.7 M.

**[0043]** A particularly preferred embodiment further comprises 20 mg/ml of proteinase K as an RNAase inactivating agent. One preferred embodiment of the lysis buffer comprises 200 μg/ml-20 mg/ml of proteinase K. Another preferred embodiment comprises 200 μg/ml -1.0 mg/ml proteinase K. Another preferred embodiment comprises 200 μg/ml - 500μg/ml proteinase K. Sodium dodecyl sulfate may also serve as the RNAase deactivating agent. Another embodiment includes 0.1-10% of 2-mercaptoethanol as an RNAase inactivating agent. One particularly preferred embodiment comprises 1% 2-mercaptoethanol. Other embodiments of RNAase inactivating agents may preferably include materials, known to those skilled in the art, that reduce disulfide bonds in RNAases.

**[0044]** Preferred embodiments of the lysis buffer further comprise chelating agents which chelate $Mg^{2+}$ and $Ca^{2+}$. One preferred embodiment comprises 0.1 mM-5 mM EDTA. A particularly preferred embodiment comprises 1 mM EDTA. Other preferred embodiments of the lysis buffer stock contain chelating agents known to those skilled in the

art including, for example and without limitation, EDTMP, 2,3-dimercaptopropanol, and EGTA.

**[0045]** Preferred embodiments of the lysis buffer may include tRNA, which may come from various sources and is included in order to inhibit non-specific absorption of blood-derived DNA and RNA to filter plates. Additionally, the presence of tRNA prevents degradation of blood-derived RNA. In one preferred embodiment, the tRNA of the working lysis buffer comprises 10 mg/ml of E. coli tRNA. Other embodiments may contain tRNA from any source known to those skilled in the art.

**[0046]** Preferred embodiments of the lysis buffer may include DNA from a wide variety of sources, which is added in order to inhibit non-specific absorption of blood-derived DNA and RNA to filter plates. The DNA of the working lysis buffer preferably comprises 10 mg/ml of sonicated salmon sperm DNA. In other embodiments, DNA from other organisms may be used.

**[0047]** The method involves quantification of mRNA, which in a preferred embodiment entails cDNA synthesis from mRNA and amplification of cDNA using PCR. In one preferred embodiment, the multi-well filterplate is washed with lysis buffer (150 µL/well x 3 times, manual) and wash buffer (150 µL/well x 3 times, manual or BioTek #G4). A cDNA synthesis buffer is then added to the multi-well filterplate (40 µL/well, manual or I&J #6). Axymat (Amgen AM-96-PCR-RD) can be placed on the multi-well filterplate, which is then placed on a heat block (37 C, VWR) and incubated (>90 min). The multi-well filterplate can then be centrifuged (2000 rpm, at 4 C for 1 min). PCR primers are added to a 384 well PCR plate, and the cDNA is transferred from the multi-well filterplate to the 384 well PCR plate. The PCR plate is centrifuged (2000 rpm, at 4 C for 1 min), and real time PCR is commenced (TaqMan/SYBER).

**[0048]** Another preferred embodiment of the invention involves a device for high-throughput quantification of mRNA from whole blood. The device includes a multi-well filterplate containing: multiple sample-delivery wells, a leukocyte-capturing filter underneath the sample-delivery wells, and an mRNA capture zone under the filter, which contains oligo(dT)-immobilized in the wells of the mRNA capture zone. In order to increase the efficiency of leukocyte collection, several filtration membranes can be layered together. The

multi-well plate is fitted upon a vacuum box, which is adapted to receive the plate and to create a seal between the multi-well plate and the vacuum box. In one preferred embodiment of the device, the vacuum box is adapted to receive a source of vacuum in order to perform vacuum aspiration. In another preferred embodiment, a multi-well supporter is placed in the vacuum box, below the multi-well filterplate. In another preferred embodiment of the device, a sealing gasket, which can be made from soft plastic such as silicon-based rubber, is inserted between the multi-well supporter and the multi-well filterplate.

**[0049]** Another preferred embodiment involves a kit for high-throughput quantification of mRNA from whole blood. The kit includes: the device for high-throughput quantification of mRNA from whole blood; heparin-containing blood-collection tubes; a hypotonic buffer; and a lysis buffer.

**[0050]** Another preferred embodiment involves a fully automated system for performing high throughput quantification of mRNA in whole blood, including: robots to apply blood samples, hypotonic buffer, and lysis buffer to the device; an automated vacuum aspirator and centrifuge, and automated PCR machinery.

## Examples

### Example 1

**[0051]** Various protocols of the method of the present invention were tested and used to quantify β-actin mRNA and CD4 mRNA from whole blood.

**[0052]** Three anticoagulants were tested: ACD, EDTA, and heparin, with heparin resulting in the highest percent of leukocyte retention. While Leukosorb membranes have been used for ACD blood in transfusion, approximately 15-40% of leukocytes passed through even when four layers of membranes were simultaneously used. EDTA blood was tested; the capacity and leukocyte retention was found to be similar to those for ACD. Most notably, however, was that 100% of the leukocytes in heparin blood were trapped on the Leukosorb membranes. The capture of 100% of leukocytes from heparin blood shows the reliability of quantification of mRNA using the present invention. These data indicate that the use of heparin blood is most suitable for the precise quantification of mRNA, whereas ACD blood is useful for applications requiring larger volumes of blood and less quantitative results.

**[0053]** The results of using of frozen versus fresh blood samples was compared. As indicated in **FIG 3**, more CD4 mRNA was recovered from leaked cells of fresh blood than from leaked cells of frozen samples.

**[0054]** The effectiveness of whole blood retention of glassfiber filters, as compared to retention values of PBT-based filter membranes, was also examined. As shown in Table II, glassfiber membranes accepted only 40 μL of whole blood, even when membranes were washed with hypotonic buffer (50 mM Tris, pH 7.4) to burst erythrocytes. Leukosorb filters, however, accepted significantly larger amounts of whole blood than the glassfiber filters, as indicated in Table II.

**Table II. Amplification of β-actin mRNA From Whole Blood**

| Membrane | Anticoagulant*[1] | Maximum blood volume, μl/well | WBC retention (%) | Subject (n) | Tested blood volume, μl/well | $x10^{-5}$ng ($CV^{+5}$,%) | CT*[3] ($CV^{*4}$,%) |
|---|---|---|---|---|---|---|---|
| Glass fiber | A, E, H | 40 | 100 | #1 (9) | 20 (A)*[2] | 4.84 ± 2.80 (57.9) | 38.0 ± 0.876 (2.31) |
| | | | | #2 (9) | 20 (A) | 4.02 ± 2.52 (62.7) | 38.4 ± 0.994 (2.59) |
| Leukotrap | A, E | 3000 | 60-85 | #1 (9) | 1000 (A) | 169 ± 112 (66.0) | 33.1 ± 1.24 (3.75) |
| | | | | #2 (9) | 1000 (A) | 100 ± 72.1 (72.0) | 30.6 ± 1.42 (4.64) |
| | H | 200 | 100 | #1 (9) | 100 (H) | 18.7 ± 4.03 (21.6) | 39.2 ± 0.414 (1.01) |
| | | | | #2 (9) | 100 (H) | 15.7 ± 3.67 (23.4) | 37.8 ± 0.432 (1.06) |
| | | | | #3 (9) | 100 (H) | 15.8 ± 3.91 (24.7) | 37.7 ± 0.379 (1.01) |
| | | | | #4 (9) | 100 (H) | 15.3 ± 4.39 (28.7) | 37.7 ± 0.429 (1.14) |
| | | | | #5 (9) | 100 (H) | 13.5 ± 3.31 (24.5) | 37.9 ± 0.455 (1.20) |
| | | | | #6 (9) | 100 (H) | 20.1 ± 4.79 (23.8) | 37.3 ± 0.365 (0.98) |

*1: A: ACD, E: EDTA, H: heparin
*2 (A, H) represents anticoagulants used in the experiments.
*3 (CT: Threshold Cycle
*4 CV: Coefficient of variation

**[0055]** Various numbers of washes with hypotonic buffer were applied to remove erythrocytes and other blood components. As indicated in **FIG 4**, washing the samples with hypotonic buffer at least three times more than doubled the amount of CD4 mRNA that was captured as compared to no washing. **FIG 4** also shows that washing the blood twelve times with hypotonic buffer resulted in the capture of the most mRNA. Additionally, various methods of vacuuming, centrifuging, and washing with ethanol followed by vacuuming blood samples to collect leukocytes were compared with respect to final CD4 mRNA quantification. **FIG 5** indicates that while vacuum aspiration resulted in better CD4 mRNA

quantification than centrifugation, washing blood samples with ethanol prior to vacuum aspiration yields the most mRNA.

**[0056]** Once the leukocytes were trapped on the glassfiber or Leukosorb membranes, various numbers of washes with hypotonic buffer were applied to remove erythrocytes and other blood components. To release mRNA from the trapped leukocytes, lysis buffer (RNA*ture*) was applied to the filterplates (40 µL/Well), and the plates were incubated at room temperature for 20 minutes. In a preferred embodiment, amplification primers are included in the lysis buffer. **FIG 6** indicates that lysis buffer plays an important role in RNase inhibition; eosinophils are filled with RNAases, which are inactivated by the lysis buffer. The multi-well filterplate was then sealed in a plastic bag and centrifuged (IEC MultiRF, 2000 rpm, at 4 C, for 1 min). Lysis buffer was then added again (20 µL/well), followed by centrifugation (IEC MultiRF, 3000 rpm, at 4 C, for 5 min). The multi-well filterplate was then removed from the centrifuge and incubated at room temperature for two hours to allow hybridization of poly(A)+ RNA tails with the immobilized oligo(dT). The multi-well filterplates were then washed three times with 150 µL Lysis Buffer to remove remaining ribonucleases, followed by three washes with 150 µL Wash Buffer (BioTek #G4) to remove the Lysis Buffer, which contained some inhibitors of cDNA synthesis.

**[0057]** Upon the final wash, the Wash Buffer was completely removed from the multi-well filterplates, and cDNA was synthesized in each well by adding 40 µL of premixed cDNA buffer. The cDNA buffer consists of: 5x First Strand Buffer (Promega M531A, 10 mM dNTP (Promega stock, 20x)), Primer (5 µM, #24), RNasin (Promega N211A, 40 U/µL), M-MLV reverse transcriptase (Promega M170A, 200 U/µL), and DEPC water. **FIG 7** indicates that the optimal concentration of MMLV for mRNA quantification is 50 units/well.

**[0058]** Axymat (Amgen AM-96-PCR-RD) was placed on the multi-well filterplate, which was then placed on a heat block (37 C, VWR) and incubated (>90 min). The multi-well filterplate was then centrifuged (2000 rpm, at 4 C for 1 min). PCR primers were added to a 384-well PCR plate, and the cDNA was transferred from the multi-well filterplate to the 384-well PCR plate. **FIG 8** indicates that the optimal value of cDNA for PCR is approximately 2 µL/well. The PCR plate was centrifuged (2000 rpm, at 4 C for 1 min), and real time PCR was commenced (TaqMan/SYBER). The method of the current

invention has high mRNA specificity; amplification of CD4 mRNA with TaqMan qPCR resulted in undetectable DNA contamination (<10 copies/well).

**[0059]** As indicated in **FIG 9**, the present invention results in low coefficients of variation for mRNA quantification. Hybridization for two hours resulted in a coefficient of variation of less than 13%, as compared to traditional coefficients of variation of approximately 300% for mRNA quantification. Moreover, as indicated in **FIG 10**, the linear results show that the amount of mRNA that is captured is directly proportional to the volume of whole blood used per well, making the present invention a reliable and reproducible method of quantifying mRNA.

Example 2

**[0060]** Fifty μL of heparinized frozen human blood was applied to Leukosorb filterplate. After each well was washed by vacuum with 150 μL of 5 mM Tris pH 7.4, 12 times followed by 150 μL of 100% ethanol once, 40 μL of Lysis Buffer, which contains 1.707-1.856 M guanidine thiocyanate, was added to the well. After incubation at room temperature for 15 min, the filterplate was placed onto the GenePlate and centrifuged with 2000 rpm at 4°C for 1 min. Additional 20 μL Lysis Buffer was added and centrifuged again for 5 min. After the GenePlate was incubated at room temperature for 2 hours, each well was washed with 100 μL Lysis Buffer 3 times, followed by 150 μL Wash Buffer (10 mM Tris, pH 7.4, 1 mM EDTA, pH 8.0, 0.5 M NaCl) 3 times. The cDNA was synthesized in the GenePlate, and 2 μL cDNA was used for TaqMan assay to quantitate CD4. The results are indicated in **FIG 11**.

Example 3

**[0061]** Fifty μL of heparinized frozen human blood was applied to Leukosorb filterplate. After each well was washed by vacuum with 150 μL of 5 mM Tris pH 7.4, 12 times followed by 150 μL of 100% ethanol once, 40 μL of Lysis Buffer, which contains 1.791 M guanidine thiocyanate with 0-0.5 mg/ml proteinase K, was added to the well. After incubation at room temperature for 15 min, the filterplate was placed onto the GenePlate and centrifuged with 2000 rpm at 4°C for 1 min. Additional 20 μL Lysis Buffer was added and centrifuged again for 5 min. After the GenePlate was incubated at room temperature for 2

hours, each well was washed with 100 μL Lysis Buffer 3 times, followed by 150 μL Wash Buffer (10 mM Tris, pH 7.4, 1 mM EDTA, pH 8.0, 0.5 M NaCl) 3 times. The cDNA was synthesized in the GenePlate, and 2 μL cDNA was used for TaqMan assay to quantitate CD4. The results are indicated in **FIG 12**.

Example 4

Lysis Buffer stock
0.5% N-Lauroylsarcosine
4X SSC
10 mM Tris HCl, pH 7.4
1 mM EDTA
0.1% IGEPAL CA-630
1.791 M guanidine thiocyanate

Working Lysis Buffer

| | |
|---|---|
| Lysis Buffer stock | 1 ml |
| 2-mercaptoethanol | 10 μL |
| Sonicated salmon sperm DNA (10 mg/ml) | 10 μL |
| E. coli tRNA (10 mg/ml) | 10 μL |
| Proteinase K (20 mg/ml stock) | 25 μL (final 0.5 mg/ml) |